Chemico-Biological Interactions 218 (2014) 107–114

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Sydnone SYD-1 affects the metabolic functions of isolated rat hepatocytes

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ARTICLE INFO

Article history:
Received 7 March 2014
Received in revised form 3 May 2014
Accepted 5 May 2014
Available online 15 May 2014

Keywords:
Sydnones
SYD-1
Hepatocytes
Respiration
Metabolism

ABSTRACT

Previously, we demonstrated that sydnone SYD-1 (3-[4-chloro-3-nitrophenyl]-1,2,3-oxadiazolium-5-olate) impairs the mitochondrial functions linked to energy provision and suggested that this effect could be associated with its antitumor activity. Herein, we evaluated the effects of SYD-1 (25 and 50 μM) on rat hepatocytes to determine its cytotoxicity on non-tumor cells. SYD-1 (25 and 50 μM) did not affect the viability of hepatocytes in suspension after 1–40 min of incubation. However, the viability of the cultured hepatocytes was decreased by ~66% as a consequence of treatment with SYD-1 (50 μM) for 18 h. Under the same conditions, SYD-1 promoted an increase in the release of LDH by ~19%. The morphological changes in the cultured cells treated with SYD-1 (50 μM) were suggestive of cell distress, which was demonstrated by the presence of rounded hepatocytes, cell fragments and monolayer impairment. Furthermore, fluorescence microscopy showed an increase in the annexin label after treatment with SYD-1 (50 μM), suggesting that apoptosis had been induced in these cells. SYD-1 did not affect the states of respiration in the suspended hepatocytes, but the pyruvate levels were decreased by ~36%, whereas the lactate levels were increased by ~22% (for the 50 μM treatment). The basal and uncoupled states of respiration of the cultured hepatocytes were inhibited by ~79% and ~51%, respectively, by SYD-1 (50 μM). In these cells, SYD-1 (50 μM) increased the pyruvate and lactate levels by ~84% and ~16%, respectively. These results show that SYD-1 affects important metabolic functions related to energy provision in hepatocytes and that this effect was more pronounced on cells in culture than those in suspension.

1. Introduction

Mesoionic compounds possess several biological activities that are primarily associated with their chemical structure [1,2]. These compounds are characterized by positively and negatively charged regions associated with their polyheteroatomic system that enables them to interact with biomolecules such as DNA and proteins. In addition, their net neutral character enables them to cross biological membranes. The sydnones were the first class of mesoionic compounds synthesized, and they have been associated with antibacterial [3,4], antitumor [5–7] and antiepileptic activities [8], among others. In addition, certain sydnones are able to scavenge free radicals. Sydnone SYD-1 (3-[4-chloro-3-nitrophenyl]-1,2,3-oxadiazolium-5-olate) (Fig. 1) has shown important cytotoxic and antitumor effects *in vivo* [7]. However, the molecular

Abbreviations: ASA, acetylsalicylic acid; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide; TRIS, tris(hydroxymethyl)-aminomethane; DMEM, Dulbecco's modified Eagle's medium; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; FITC, fluorescein isothiocyanate; EGF, epidermal growth factor; HBSS, Hanks balanced salt solution; PBS, phosphate buffered saline; PI, propidium iodide; SYD-1, 3-[4-cloro-3-nitrophenyl]-1,2,3-oxadiazolium-5-olate; MPT, mitochondrial pore transition; DAF-FM, 4-amino-5-(N-methylamino)-3′,6′-bis(acetyloxy)-2′,7′-difluoro-spiro[isobenzofuran-1(3H),9′-[9H]xanthen]-3-one, diaminofluorescein-FM diacetate.

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http://dx.doi.org/10.1016/j.cbi.2014.05.002
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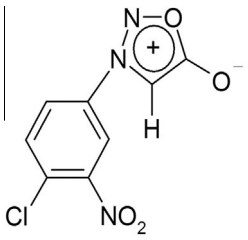

Fig. 1. Chemical structure of 3-[4-chloro-3-nitrophenyl]-1,2,3-oxadiazolium-5-olate (SYD-1).

mechanisms involved in these effects are not known. Because the mitochondria are involved in the mechanisms of cell death induction, we previously demonstrated that SYD-1 depresses the efficiency of oxidative phosphorylation and suggested that this could be related to its antitumor activity [9]. Recently, we also showed that SYD-1 inhibits the processes associated with oxidative stress, specifically the iron-induced lipoperoxidation, the oxidation of pyridine nucleotides and the mitochondrial pore transition [10]. In the same study, the $O_2^{\cdot-}$ scavenging ability of SYD-1 was also shown. Because it is especially important that antineoplastic drugs are specific to tumor cells, the significant antitumor activity of SYD-1 and the absence of studies about its effects on non-tumor cells prompted this evaluation of the effects of SYD-1 on the metabolic functions of isolated rat hepatocytes.

2. Materials and methods

2.1. Chemicals

D-Mannitol, HEPES, rotenone, FCCP, oligomycin, MTT, dexamethasone, collagenase types IV and IA, glucagon, insulin, epidermal growth factor (EGF), trypsin, glutamine, penicillin, streptomycin and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest commercially available purity. SYD-1 was synthesized by the Department of Chemistry at the Federal Rural University of Rio de Janeiro, Brazil, and its structure was confirmed by ^{1}H NMR, ^{13}C NMR and mass spectrometry. For use in this work, SYD-1 was dissolved in DMSO and further diluted with the assay medium. To validate each assay, duplicate controls with DMSO were included at the concentrations used in the experiments. The DMSO had no effect on the parameters analyzed.

2.2. Animals

Male Wistar rats (180–200 g) were obtained from the Central Animal House of the Federal University of Paraná (PR, Brazil). The animals were housed at 22 ± 1 °C under a 12 h-light/12 h-dark cycle (lights turned on at 8 am) and had free access to standard laboratory food (Purina®) and tap water. The experiments were conducted following the recommendation of Brazilian Law 6638, 05/11/1979 for the scientific management of animals, and the procedures were approved by the Institutional Animal Ethics Committee (certificate number 548).

2.3. Isolation, culture and treatment of hepatocytes

The hepatocytes were obtained by monovascular liver perfusion of Wistar rats, as described previously by [11,12] with modifications. The male rats were weighed and anesthetized intraperitoneally with a mixture of ketamine (60 mg/kg) and xylazine (7.5 mg/kg). Following laparotomy, 100 μL of sodium heparin (5000 U/mL) were injected into the abdominal cava vein. The portal and thoracic cava veins were cannulated, and the liver was perfused with Krebs solution (2.399 M NaCl, 96 mM KCl, 24 mM KH_2PO_4, 24 mM $MgSO_4$, 480 mM $NaHCO_3$ and 1 M HEPES buffer, pH 7.4) containing 1.3 M $CaCl_2$, 20 mg collagenase (types IA and IV) and carbogen (95% O_2:5% CO_2). The liver was excised, and the cells were released by mechanical action, filtered through 50 μM nylon membranes and centrifuged at 400 rpm for 5 min at 4 °C. Subsequently, the cells were centrifuged four times with Krebs solution supplemented with 20% bovine serum albumin (BSA) and treated with carbogen. The cells were suspended in high glucose DMEM supplemented with bovine fetal serum (10%), insulin (100 nM), glucagon (10 nM), EGF (10 ng/mL), dexamethasone (50 nM), penicillin (100 U/mL) and streptomycin (100 ng/mL). Cell viability was determined using the Trypan blue (0.4%, w/v) exclusion method as previously described by Philips [13]. Only the cell suspensions with viabilities higher than 80% were plated (1×10^6 cells/plate on a 60 mm plate) and cultured for further experiments. Four hours after plating, the medium was replaced by Hepatozyme® with or without SYD-1 (25 or 50 μM) or DMSO (0.01%), and incubated for 18 h prior to the use in the assays.

2.4. Cellular viability assays

The viability of the suspended hepatocytes incubated with SYD-1 (25 or 50 μM) for 1, 2, 20 and 40 min was evaluated using the Trypan blue (0.4%, w/v) exclusion method as previously described by Philips [13]. The hepatocytes (1×10^6 cells) were seeded in a 60 cm^2-well plate with Hepatozyme® medium with or without SYD-1 (25 or 50 μM) for 18 h. After incubation, the medium was discarded and a tetrazolium dye (MTT) solution (0.5 mg/mL) was added. The plates were allowed to incubate at 37 °C for 3 h. The MTT-formazan crystals that formed were dissolved in DMSO and the absorbance was measured at 540 nm using a microplate reader [14]. Under the same conditions, the viability of the hepatocytes was also determined by the release of LDH into the culture medium using an LDH assay kit (Labtest®, Lagoa Santa, Brazil) according to the manufacturer's recommended protocol. After SYD-1 treatment, the supernatant was collected and centrifuged at 1200 rpm for 10 min, and the LDH activity was measured. The results were expressed as LDH activity (U/L).

2.5. Morphological analysis

The morphological changes of the hepatocytes after the treatments were examined with an Axiovert 40CSFL inverted microscope with 10× magnification.

2.6. Annexin V-FITC and propodium iodide double staining

Fluorescence microscopy was performed to determine the cell apoptosis or necrosis induced by SYD-1. The cells (1×10^6 cells/mL) were seeded and incubated with SYD-1 (25 and 50 μM) at 37 °C for 24 h. Then, the medium was exchanged for binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) containing 5 μM annexin V-FITC and the cells were incubated for 10 min. Finally, PI (0.08 μM) was added and images were immediately captured with an Axiovert 40CSFL inverted microscope with 10× magnification.

2.7. Determination of lactate and pyruvate

These assays were performed with both suspended and cultured hepatocytes. For the suspended cells, the hepatocytes (1×10^6 cells/mL) were maintained in PBS and incubated for 2 min with SYD-1 (25 and 50 μM) at 37 °C under continuous shaking. Then, glucose (5 mM) was added, and samples were collected at 1, 20 and 40 min. The samples were immediately centrifuged

(1000 rpm for 1 min), and the concentrations of lactate and pyruvate were determined in the supernatant. Additionally, the lactate and pyruvate concentrations in the cultured cells were obtained from the supernatant of the hepatocyte cultures after incubation with SYD-1 (25 or 50 μM) for 18 h. The supernatant was collected and centrifuged (1000 rpm for 1 min), and the lactate and pyruvate concentrations were measured [15,16].

2.8. Oxygen uptake

The respiration of the intact hepatocytes was measured by high-resolution respirometry with an Oxygraph-2k (OROBOROS INSTRUMENTS, Innsbruck, Austria) using two chambers at 37 °C under gentle agitation. The oxygen solubility factor was 0.89 for the DMEM and, at local barometric pressure (91 kPa), the concentration of oxygen at air saturation was 172.42 μM. The oxygen uptake of the freshly isolated hepatocytes in suspension (1×10^6 - cells) was monitored in DMEM after incubation for 2 min with SYD-1 (25 or 50 μM). For the measurement of respiration in the cultured hepatocytes treated with SYD-1 (25 or 50 μM for 18 h), the cells were detached from the culture dishes with a trypsin solution and suspended (1×10^6 cells) in DMEM. The oxygen consumption was rated in the different states of respiration as previously described [17–21], and the states were defined as: basal, oxygen consumption in the absence of inhibitors or uncouplers; leak, respiration in the presence of oligomycin (2 μg/mL); and uncoupled, oxygen consumption in the presence of the uncoupler FCCP (0.5 μM). The oxygen flow in these states was corrected by the subtraction of non-mitochondrial respiration, which was obtained after the addition of rotenone (0.5 μM) and antimycin (3 μg/mL). The results are expressed as the oxygen flow per cells [pmol/(seg * 1×10^6 cells)] as media ± SD.

2.9. Protein determination

The protein concentrations were determined using the method described by Lowry with BSA as the standard [22].

2.10. Statistical analysis

The relevant statistical analysis was performed by analysis of variance (ANOVA) and by Tukey's test for average comparison. The results were expressed as the standard error ± the SEM, and the values were considered significant when $P < 0.05$.

3. Results

3.1. Cytotoxic effects of SYD-1

SYD-1 (25 and 50 μM) did not affect the viability of hepatocytes in suspension after 1–40 min of incubation, which was determined by the Trypan blue assay (data not shown). Two different assays were used to evaluate the viability of the cultured hepatocytes, MTT reduction by dehydrogenases from viable cells and LDH release resulting from cell membrane damage [23,24]. The results of the MTT assays (Fig. 2A) showed that treatment for 18 h with SYD-1 at the lowest concentration (25 μM) did not affect hepatocyte viability; however, at the highest concentration (50 μM), the sydnone was able to decrease cell viability by up to ~66%. Under the same conditions, SYD-1 promoted an increase in the release of LDH by ~19% only at the highest concentration (50 μM; Fig. 2B).

3.2. Effects of SYD-1 on hepatocyte morphology

The freshly isolated hepatocytes were morphologically intact, as visualized by Trypan blue, exhibiting a rounded shape (data not shown). After adhering to the culture plates, these cells acquired the typical cubic shape often with two nuclei and formed a monolayer (Fig. 3A – control). The solvent for SYD-1 (0.01% DMSO) did not affect the morphology of hepatocytes (Fig. 3B). SYD-1 at the lowest concentration (25 μM – Fig. 3C) was unable to change the organization of monolayer; however, some detached rounded cells were present, suggesting cell distress. The presence of detached cells was increased at the highest concentration of SYD-1 (50 μM), accompanied by some cell fragments and the disruption of the cell monolayer in localized areas (Fig. 3D). Although less pronounced, these results are in agreement with the effects of SYD-1 on hepatocyte viability (Fig. 2A and B), which demonstrated that the compound was cytotoxic at the highest concentration.

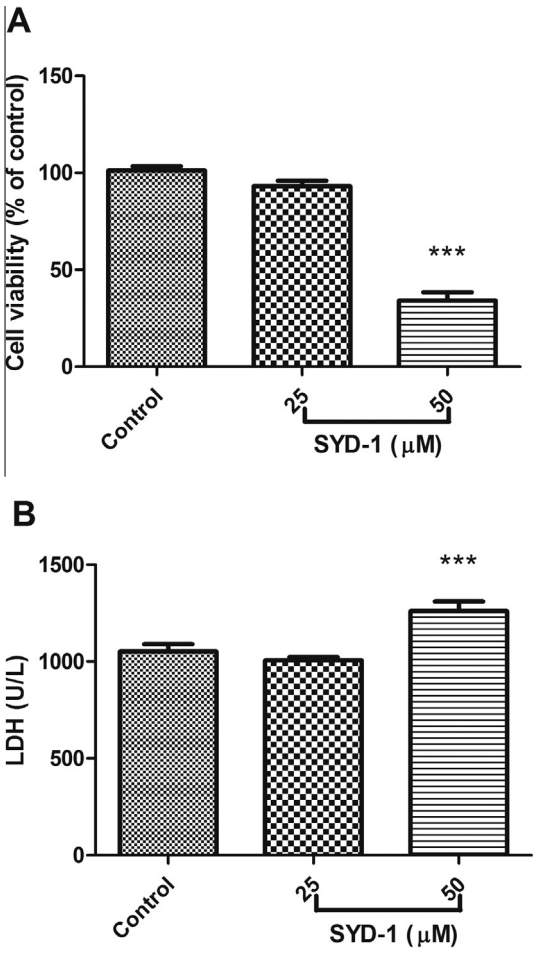

Fig. 2. Cytotoxic effects of SYD-1. The experimental conditions are described in Section 2. (A) MTT assay. The cells (1×10^6) were seeded in a 60 mm-well plate with or without SYD-1 at concentrations of 25 or 50 μM for 18 h. Then, a tetrazolium dye (MTT) solution (5 mg/mL) was added. The plates were incubated at 37 °C for 3 h. The MTT-formazan crystals that formed were dissolved in DMSO, and the absorbance was measured at 540 nm using a microplate reader. The results were expressed as the viability of the control (%). * and *** denote values significantly different from the control at $P < 0.05$ and $P < 0.0001$, respectively. (B) LDH releasing assay. Under the same conditions as the previous assay, after SYD-1 treatment, the supernatant was collected and centrifuged at 1200 rpm for 10 min. The LDH activity was measured using an LDH assay kit (Labtest) according to the manufacturer's recommended protocol. The results were expressed as LDH activity (U/L). *** denotes values significantly different from the control at $P < 0.0001$.

3.3. Effect of SYD-1 on the induction of hepatocyte death

Because SYD-1 (50 μM) was able to reduce the viability of cultured hepatocytes (Figs. 1 and 2), assays using annexin and PI were

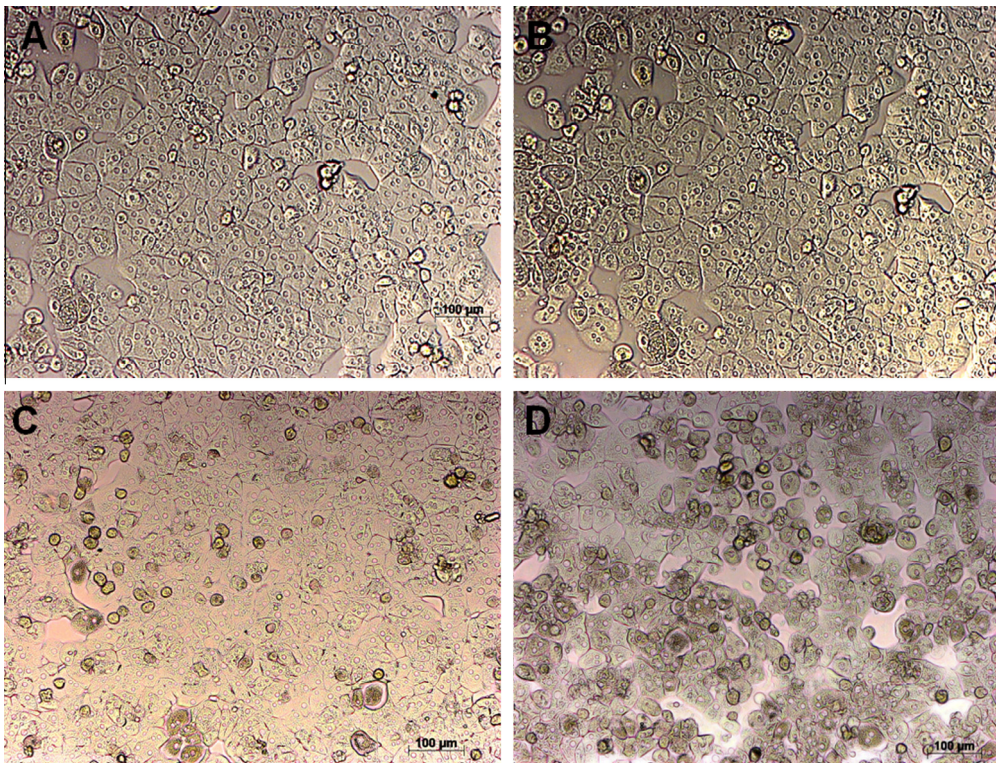

Fig. 3. Effects of SYD-1 on hepatocyte morphology. The experimental conditions are described in Section 2. The rat primary hepatocytes (1×10^6 cells) were incubated with SYD-1 (25 or 50 µM) for 18 h. The images were obtained using inverted microscopy and are representative of three independent experiments. The scale shows the real magnification. (A) Control (untreated cells); (B) DMSO (cells treated with 0.01% DMSO); (C) cells treated with 25 µM SYD-1; (D) cells treated with 50 µM SYD-1.

performed to identify the pathway by which the compound induces the cell death. In these assays, ASA (20 mM) was used as a positive control of cell death by apoptosis (Fig. 4A). The images demonstrate that after treatment with the highest concentration of SYD-1 (50 µM) the fluorescence of annexin increased relative to the control (Fig. 4D-II and B-II, respectively). The same effect was not observed after treatment with SYD-1 at the lowest concentration (25 µM; Fig. 4C-II). The increase was also not visualized in PI fluorescence after treatment with the compound at both concentrations (Fig. 4C-III and D-III). These results suggest that SYD-1 induces hepatocyte death by apoptosis and are in accordance with the results of the viability and morphological assays (Figs. 2 and 3), which showed the most pronounced effect of the mesoionic compound at 50 µM.

3.4. Effect of SYD-1 on hepatocyte respiration

Because SYD-1 decreases the viability and promotes morphological changes of hepatocytes, it became important to evaluate whether the compound would also be able to affect the metabolic functions of these cells. To explore this possibility, the hepatocytes in suspension and in culture were treated with SYD-1 for 2 min and 18 h, respectively, and the parameters of cell respiration were determined. These assays were performed with cells that were not yet permeabilized to approximate the experimental and physiological conditions. Consequently, the reagents used were membrane-permeable inhibitors or uncouplers of mitochondrial phosphorylation. The oxygen uptake was evaluated in the four states of respiration [17–21]: basal, leak, uncoupled and inhibited. The results are shown in Fig. 5, where the solid line represents the oxygen concentration (µM) and the dashed line represents the oxygen flow ([pmol/(seg * 1×10^6 cells)]. In Fig. 5, the *basal* oxygen consumption (in the absence of inhibitors or uncouplers) was significantly decreased by oligomycin. The oxygen uptake in the presence of oligomycin results from the re-entry of protons into the mitochondrial matrix, characterizing the *leak state*. The addition of a classical uncoupler, FCCP, promotes a significant increase in oxygen consumption (*uncoupled state*). Finally, mitochondrial respiration was completely inhibited by the addition of rotenone and antimycin (*inhibited state*). The values of oxygen flow corresponding to the *inhibited state* were subtracted from the other states of respiration so that only the oxygen uptake resulting from mitochondrial respiration was represented in each state [17–21]. The results of the assays with the hepatocytes in suspension and in culture were analyzed by the DataLab4® software and are shown in Fig. 6. The sydnone SYD-1 (25 and 50 µM) did not affect the states of respiration of the suspended hepatocytes (Fig. 6A). For the cultured hepatocytes, SYD-1 affects the cellular respiration only at the highest concentration (50 µM), and it decreased the oxygen uptake by ~79% and ~51% for the basal and uncoupled states, respectively (Fig. 6B). These results corroborate those from the viability and morphological assays (Figs. 2 and 3), which demonstrated the most pronounced effect of the mesoionic compound at a concentration of 50 µM.

3.5. Effect of SYD-1 on lactate and pyruvate release

The inhibition of respiration by SYD-1 suggests that it could impair mitochondrial ATP production, which could also result in the activation of glycolytic pathway. To evaluate this possibility, the levels of pyruvate and lactate released by these cells were measured. For the suspension cells, the lactate and pyruvate levels were determined in the incubation medium at 1, 20 and 40 min after a previous incubation with SYD-1 (25 and 50 µM) for 2 min. The reaction was initiated by the addition of 5 mM glucose. The results are shown in Fig. 7, which shows a decrease in the pyruvate

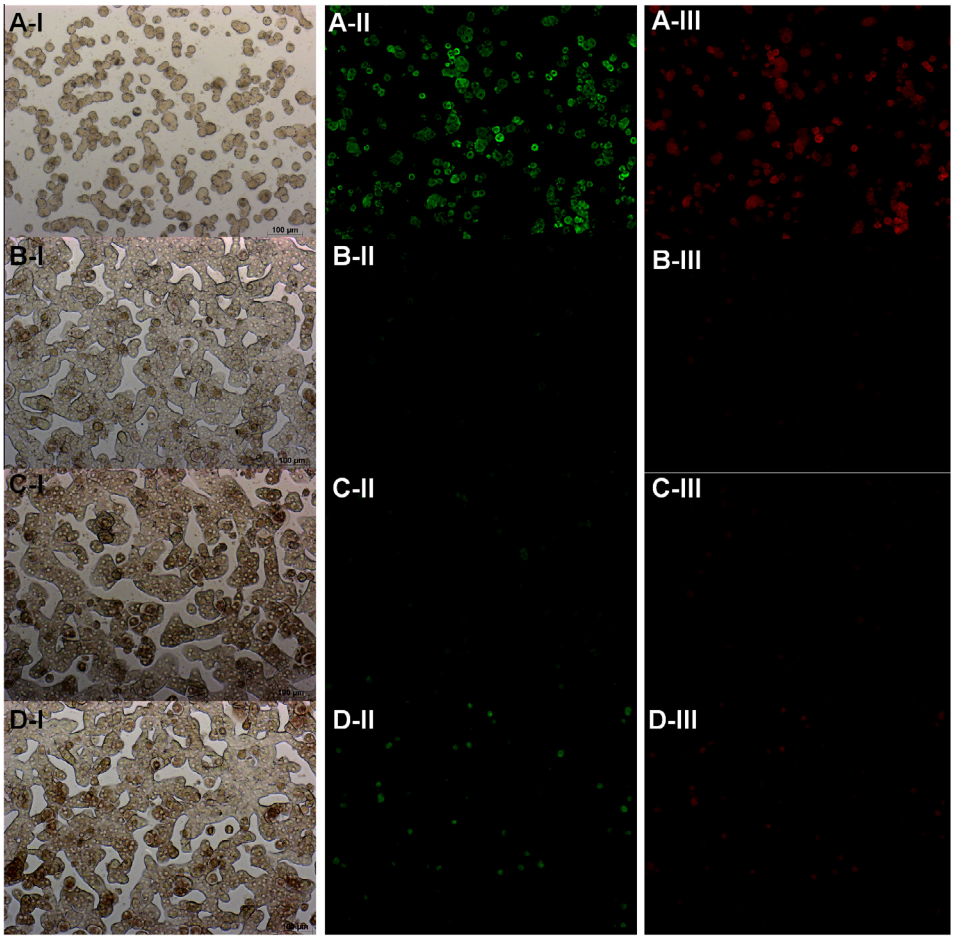

Fig. 4. Annexin V-FITC and propidium iodide staining of hepatocytes treated with SYD-1. The experimental conditions are described in Section 2. The rat primary hepatocytes (1×10^6 cells) were incubated with SYD-1 (25 or 50 μM) for 18 h. The images were captured with an AXIOVERT 40CSFL fluorescence microscope and are representative of three independent experiments. The scale shows the real magnification. The annexin V-FITC-positive cells are stained green and the PI-positive cells are stained red. (A) Positive control – cells treated with 20 mM ASA; (B) control – cells treated with 0.01% DMSO; (C) cells treated with 25 μM SYD-1; (D) cells treated with 50 μM SYD-1. I – bright phase; II – annexin V-FITC; and III – propidium iodide. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

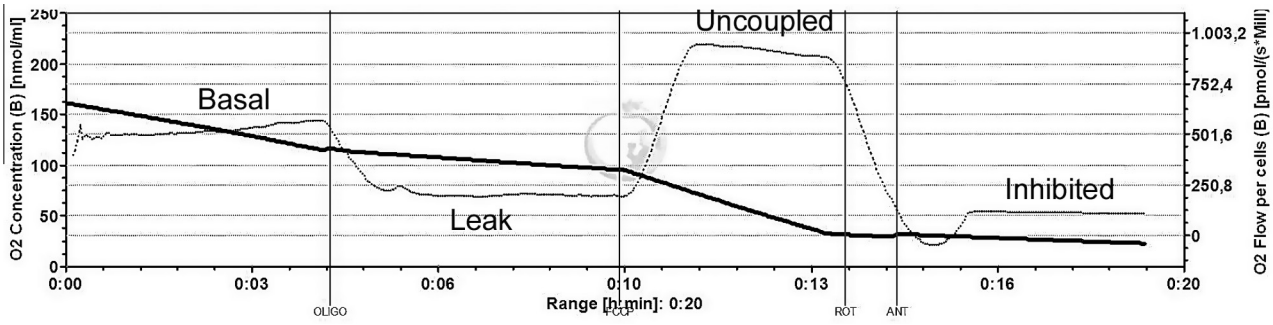

Fig. 5. Representative trace of hepatocyte respiration in an OROBOROS-2K oxygraph. The experimental conditions are described in Section 2. Legends: oligo – oligomycin; ROT – rotenone; ANT – antimycin. Solid line: oxygen concentration; dashed line: oxygen flow.

levels by ~15%, ~25% and ~27% at 1, 20 and 40 min, respectively, when SYD-1 was present at 25 μM. At the highest concentration (50 μM), this effect was more pronounced, with a ~27% decrease in the pyruvate levels at 1 min and a ~36% decrease at 20 and 40 min (Fig. 7A). Conversely, the lactate levels were increased; however, this effect was not observed immediately (1 min). This increase was approximately 21% and 27% after a 20 min incubation with SYD-1 (25 μM and 50 μM, respectively). For the highest concentration (50 μM), a ~18% increase was observed after a 20 min incubation and a ~22% increase was observed after a 40 min incubation (Fig. 7B).

In the cultured hepatocytes, SYD-1 promoted an increase in both pyruvate (~84%; Fig. 7C) and lactate (~16%; Fig. 7D) levels, but only at the highest concentration (50 μM). Taken together, these results suggest that the mesoionic SYD-1 compound causes the activation of anaerobic glycolysis in these cells. This effect is

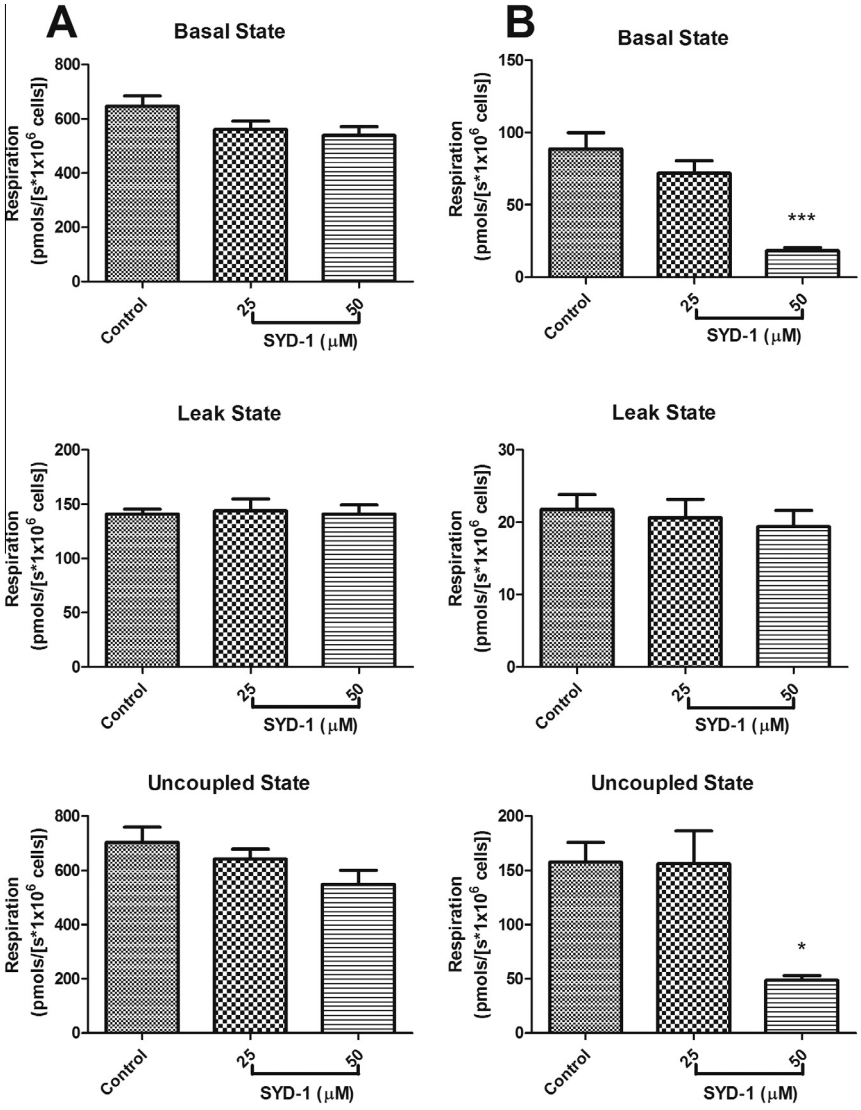

Fig. 6. Effect of SYD-1 on the oxygen uptake of hepatocytes. (A) Hepatocytes in suspension. (B) Hepatocytes in culture. The experimental conditions are described in Section 2. The cells (1×10^6) were transferred to the Oroboros 2-K oxygraph chambers, and the oxygen consumption was determined in the absence of inhibitors or uncouplers (basal state), in presence of oligomycin (leak state) and in the presence of FCCP (uncoupled state). The values represent the mean ± the standard error of six different experiments. * and *** denote values significantly different from the control at $P < 0.05$ and $P < 0.0001$, respectively.

consistent with the inhibition of oxygen consumption observed for the highest concentration of SYD-1 only.

4. Discussion

The significant antitumor and cytotoxic effects of SYD-1 have motivated our studies to elucidate its mechanism of action. We previously showed that SYD-1 impairs the mitochondrial function linked to energy provision and suggested that this effect could be involved in its antitumor activity [9]. Recently, we also demonstrated that the SYD-1 inhibits processes associated with oxidative stress [10]. Despite the potential use of SYD-1 as an antitumor agent, its effects on non-tumor cells were not yet ascertained. Therefore, in this study, we used rat hepatocytes as model of non-tumor cells to evaluate the cytotoxicity of SYD-1. Concentrations of 25 and 50 μM were used based on previous experiments with mitochondria [9,10].

Our experimental strategy included assays with freshly isolated hepatocytes (in suspension) and cultured hepatocytes. With the hepatocytes in suspension, SYD-1 did not affect the viability and respiration for either concentration at all times measured in the assays (1–40 min). The absence of any effect on hepatocyte respiration would be unexpected based on the significant inhibition of state 3 in isolated mitochondria that is promoted by SYD-1 [9]. However, the methodological differences must be considered. The hepatocyte respiration assays were performed without cell permeation or the addition of exogenous substrate. It is possible that, in this condition, the access of SYD-1 to the mitochondria was impaired and therefore, significant effects on the respiratory parameters were not observed. However, the pyruvate and lactate release from the cells was affected by SYD-1 (25 and 50 μM). The increased levels of lactate (after 20 and 40 min of incubation) and the decreased levels of pyruvate (starting after 1 min of incubation) suggest an activation of anaerobic glycolysis (Fig. 7) in response to SYD-1 treatment. We previously showed that another class of the mesoionic compounds, the 1,3,4-thiadiazolium derivatives, are able to insert into the lipid bilayers (liposomes), thus changing their fluidity [25,26]. Therefore, it is possible that

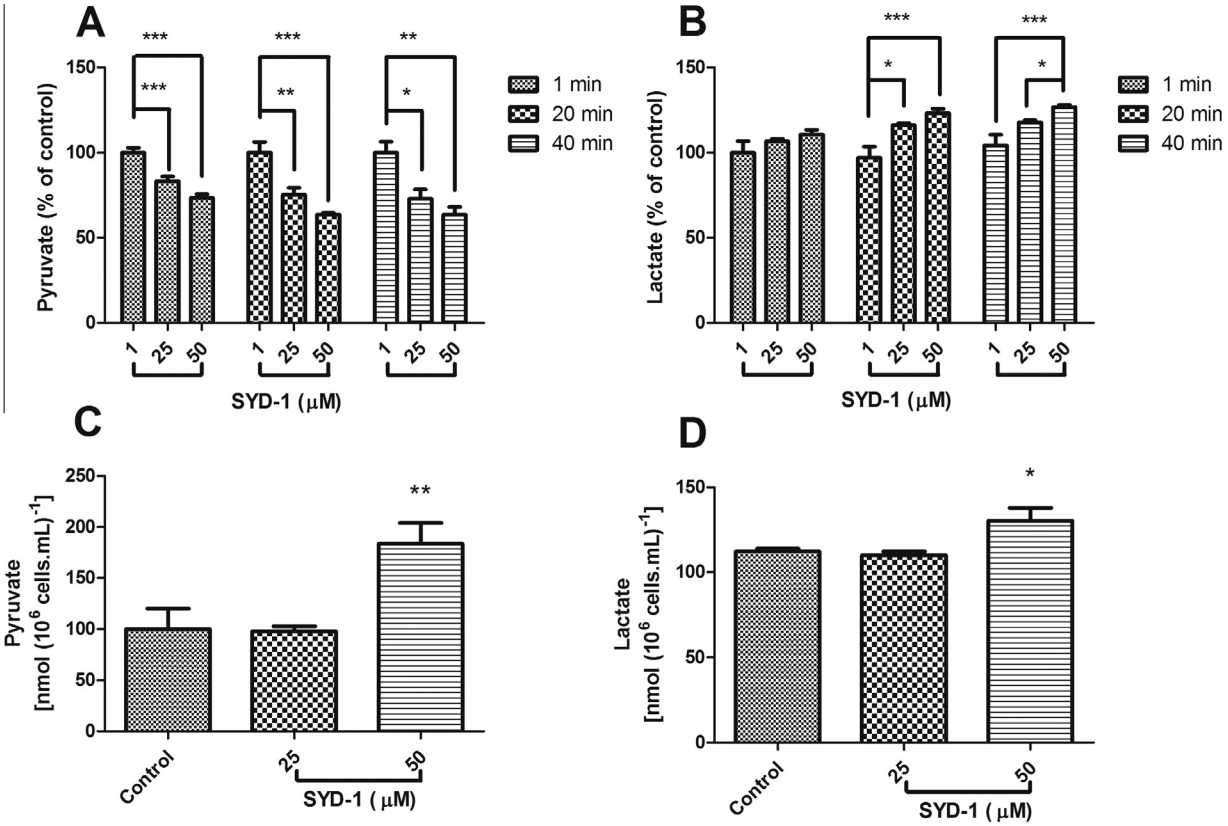

Fig. 7. Effects of SYD-1 on the pyruvate and lactate levels in hepatocytes. (A) Pyruvate levels in suspended hepatocytes; (B) lactate levels in suspended hepatocytes; (C) pyruvate levels in cultured hepatocytes; (D) lactate levels in cultured hepatocytes. The experimental conditions are described in Section 2. For A and B, the cells (1×10^6 cells/mL) were collected and maintained in PBS at 37 °C under constant shaking and were treated with SYD-1 (25 and 50 μM) for 2 min. Then, the reaction was initiated by the addition of 5 mM glucose. For C and D, the cells (1×10^6 cells/mL) were seeded and treated with SYD-1 (25 and 50 μM) for 18 h. The pyruvate and lactate concentrations were measured in the supernatant. The control was the cells in the absence of SYD-1 and in the presence of 0.01% DMSO. For pyruvate, 100% corresponds to t_1 = 29.09 ± 0.8; t_{20} = 25.67 ± 1.61; t_{40} = 25.75 ± 1.66. For lactate, 100% corresponds t_1 = 34.64 ± 2.3; t_{20} = 42.0 ± 2.78; t_{40} = 46.0 ± 3.10. The results are expressed as the mean ± the S.E.M. of five different experiments. *, ** and *** denote values significantly different from the control at $P < 0.05$, $P < 0.01$ and $P < 0.0001$, respectively.

SYD-1 crossed the cellular membrane, reaching a concentration inside the hepatocytes that was sufficient to affect the glycolytic pathway but not impair mitochondrial functions.

In the cultured hepatocytes, only the highest concentration of SYD-1 (50 μM) was cytotoxic, significantly decreasing cell viability (Fig. 2), promoting marked changes in hepatocyte morphology (Fig. 3) and decreasing the oxygen uptake in the basal and uncoupled states (Fig. 6B). These effects could all be related to the induction of apoptosis as suggested by the assays with annexin and PI (Fig. 4). Additionally, in contrast to the observations in the suspended cells, in cultured hepatocytes, SYD-1 (50 μM) promoted an increase in both pyruvate and lactate levels. The most pronounced effects of SYD-1 on the cultured hepatocytes seemed to be related to the longer treatment times with SYD-1 (18 h) when compared with the suspended cells, which were observed for a maximum of 40 min for the pyruvate and lactate assays. It is possible that, in cultured hepatocytes, SYD-1 reached a higher concentration than in the suspended cells, which resulted in more pronounced effects for all analyzed parameters.

The effects of SYD-1 on the cultured hepatocytes may also be associated with release of nitric oxide. In fact, in experiments performed in our laboratory with the fluorescent DAF-FM probe in a mitochondria-free system, we verified the release of 13.69 ± 2.67 nM of NO·/min from SYD-1 (3 mM). Also, the addition of hemoglobin (80 μM) was able to reverse the inhibition of state 3, promoted by SYD-1 (100 μM) in isolated mitochondria. In these assays the rate of oxygen flux during state 3 (537.34 ± 29.47 pmol O_2/(s *mg protein)) was significantly reduced (289.22 ± 23 pmol O_2/(s *mg protein)) by addition of 100 μM SYD-1 as previously described [9]. This inhibition of ~53% in oxygen consumption was partially reversed by hemoglobin addition (350.07 ± 17.31 pmol O_2 mg protein – 33% of inhibition in comparison to control), reinforcing that NO· is released from SYD-1 structure. Moreover, Chimenti and colleagues (2007) showed that NOR-3 (50–150 μM), a known NO• donor, decreased the viability and respiration of cultured rat hepatocytes after incubation for 2 h and 40 min, respectively [27]. Another important aspect of this work is that at the lowest concentration (25 μM) SYD-1 slightly affected the lactate and pyruvate levels in the cultured hepatocytes. Likewise, assays with SYD-1 at the same concentration in hepatocarcinoma cells (HepG2), also performed in our laboratory, showed significant effects of SYD-1 on the viability and respiratory parameters of these cells (data not shown). These results indicate a possible selectivity of SYD-1 (25 μM) for cancer cells.

In conclusion, SYD-1 (50 μM) reduces the viability, changes the morphology and impairs certain metabolic functions in cultured hepatocytes. These effects may be caused by the impairment of the mitochondrial functions linked to energy provision and are relevant to the advancement of studies proposing the use of SYD-1 as an antitumor agent.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This investigation was supported by the Brazilian research funding agencies CNPq and CAPES.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2014.05.002.

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